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The Isolation and Characterisation of the Ovine Growth Hormone Gene

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Abstract

The ovine growth hormone gene has been isolated and sequenced, together with about 1 kbp of DNA flanking each end of the gene. The structure of the gene is similar to that found for other growth hormone genes, particularly the bovine gene, and has a primary transcript of 1792 bp, with five exons, and with intron sizes of 264 bp, 231 bp, 227 bp and 273 bp.

The gene is flanked by artiodactyl-specific middle-repetitive DNA, consisting mainly of elements belonging to the 'C-A3' family of repeated DNA. A previously unreported sequence has been found, which we have named an 'E' element.

Introduction

The transfer of recombinant DNA into animals by microinjection into the pronuclei of one-cell embryos has been demonstrated in mice, pigs, rabbits and sheep (Hammer *et al.* 1985; Brem *et al.* 1986; Palmiter and Brinster 1986; Pursel *et al.* 1986; Nancarrow *et al.* 1987). This provides a technique for the manipulation of phenotypic characters which may have practical value for the farming industries. One of the most important of these is the growth rate of the animal. By introducing into mice a fusion gene consisting of a growth hormone coding sequence and the promoter sequence of a mouse metallothionein gene (Palmiter *et al.* 1982, 1983), growth rate has been altered substantially due to the elevation of circulating growth hormone levels.

The growth response of transgenic mice appears insensitive to the coding sequence of the growth hormone gene, since metallothionein promotor fusions to bovine, human or porcine growth hormone coding sequences give essentially similar increases in growth rate (Palmiter *et al.* 1982, 1983; Michalska *et al.* 1986*a*). For domestic animals, however, the coding sequence may need to encode the hormone specific to each species, since transgenic pigs which expressed the human or the bovine growth hormone did not show any increase in growth rate (Hammer *et al.* 1985; Pursel *et al.* 1986); but when they contained the porcine coding sequence, their growth rate increased by about 25% (Michalska *et al.* 1986*b*).

The species we are using for gene transfer experiments is the Australian Merino and, therefore, we have isolated and characterized the ovine growth hormone gene. Ovine growth hormone is a polypeptide of 191 amino acids, similar in structure to other members of the vertebrate growth hormone family, in particular the bovine hormone, with which it has a 98.7% homology in amino acid sequence (Wallis 1978). In this paper we have studied the structure of the ovine gene and its surrounding repeated sequence DNA elements. We have also compared it with the bovine gene, thereby defining the nature and boundaries of some of the repeated sequence elements adjacent to the bovine gene.

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Materials and Methods

Restriction enzymes, RNAase H, *Eco*RI methylase, DNA polymerase I and DNA polymerase (Klenow fragment) were obtained from Boehringer Mannheim, Australia, or New England Biolabs, U.S.A., oligo(dT)-cellulose (type 7) from P.L. Biochemicals, U.S.A., reverse transcriptase from Life Sciences, U.S.A., T4 DNA ligase, DNA nick-translation kit and [^{32}P]-labelled deoxynucleotides from BRESA, Australia, and L-[^{35}S]-methionine and [^{125}I]-labelled protein A (*Staphylococcus aureus*) from The Radiochemical Centre, Amersham, U.K. Goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate was obtained from Biorad, U.S.A. Rabbit anti-ovine growth hormone antibody was donated by A. Wallace, CSIRO, and donkey anti-rabbit serum antibody was donated by M. Radford, CSIRO.

All other chemicals were reagent grade when possible.

Preparation of RNA

Pituitaries were collected from Merino sheep and total RNA was isolated by the methods of Martial *et al.* (1977) and Ward and Kasmarik (1980). Polyadenylated RNA was prepared by affinity chromatography on oligo(dT)-cellulose (Bantle *et al.* 1976).

Preparation and Cloning of cDNA

Double-strand cDNA was prepared by standard techniques (Maniatis *et al.* 1982), using RNAase H priming for the second strand synthesis (Gubler and Hoffman 1983). The resultant cDNA was inserted into DNA of the expression phage λgt11-Amp3 (Kemp *et al.* 1983), packaged *in vitro*, and used to infect *Escherichia coli* BTA282.

Immunological and Hybridization Screening of the cDNA Library

After plating, bacterial colonies were replicated onto nitrocellulose filters and grown for 8 h at 30°C and the phage was induced by incubating for 2 h at 42°C. Cell lysis and colony immunoassay were performed by the method of Helfman *et al.* (1984), substituting 0.05% Tween-20 for Triton X-100 in the washing steps.

The antiserum to sheep growth hormone was preabsorbed to a lysate of the parental bacterial strain BTA282 containing λgt11-Amp3 (Helfman *et al.* 1984) and used directly on the filter at a dilution sufficient to detect 1 ng of purified ovine growth hormone.

Bound antibody was detected with [^{125}I]-labelled protein A (4.5 μCi per filter, 36 mCi per mg), or by enzyme immunoassay using a goat anti-rabbit immunoglobulin–horseradish peroxidase conjugate (Biorad, 1 : 18 000 dilution), using a solution of 0.5 mg/ml 4-chloro-1-naphthol and 0.01% (v/v) hydrogen peroxide for colour development.

Hybridization screening of colonies was by the method of Grunstein and Hogness (1975).

Genomic DNA Library Screening

A sheep genomic library constructed in phage λCharon 4A was screened by standard techniques (Maniatis *et al.* 1982), using a nick-translated probe consisting of the full-length sheep growth hormone cDNA. Clones containing the growth hormone sequence were mapped by restriction enzyme analysis and the gene subcloned into the plasmid pBR322 prior to sequencing.

DNA Sequencing

Cloned cDNA inserts were sequenced by the chain-terminating method of Sanger *et al.* (1977), using the M13 cloning vehicles of Messing *et al.* (1977, 1981).

Primer extension experiments were performed as described by Hudson and Davidson (1984).

Results

Isolation of the Ovine Growth Hormone Gene

In order to isolate the genomic growth hormone sequence, an ovine growth hormone cDNA was first isolated using the expression vector λgt11-Amp3, and used to screen a library of sheep genomic sequences, cloned as a partial *Eco*R1 digest in the vector λCharon 4A. Phage plaques which showed strong hybridization were purified and their DNA inserts examined. The base sequence of the gene was obtained from clone λOGH2. A restriction map for the insert of

this clone is shown in Fig. 1. The growth hormone gene was entirely located within a 3.9 kb *Bam*HI/*Hin*dIII fragment, which was subcloned into the plasmid pBR322 and mapped in finer detail. The base sequence of a 1.9 kb *Bam*HI/*Kpn*I fragment was then determined, together with about 500 bp of DNA surrounding each end of this fragment. The sequence is shown in Fig. 2.

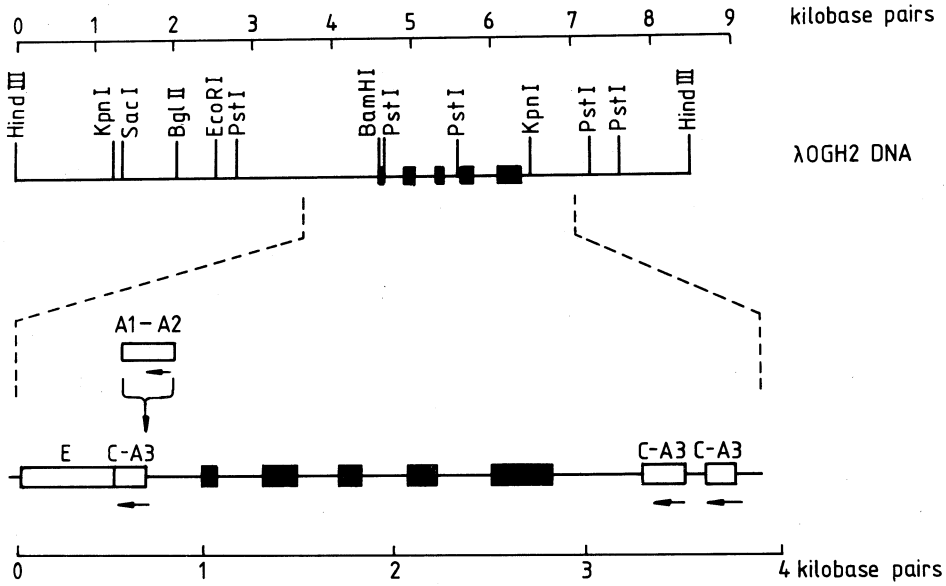
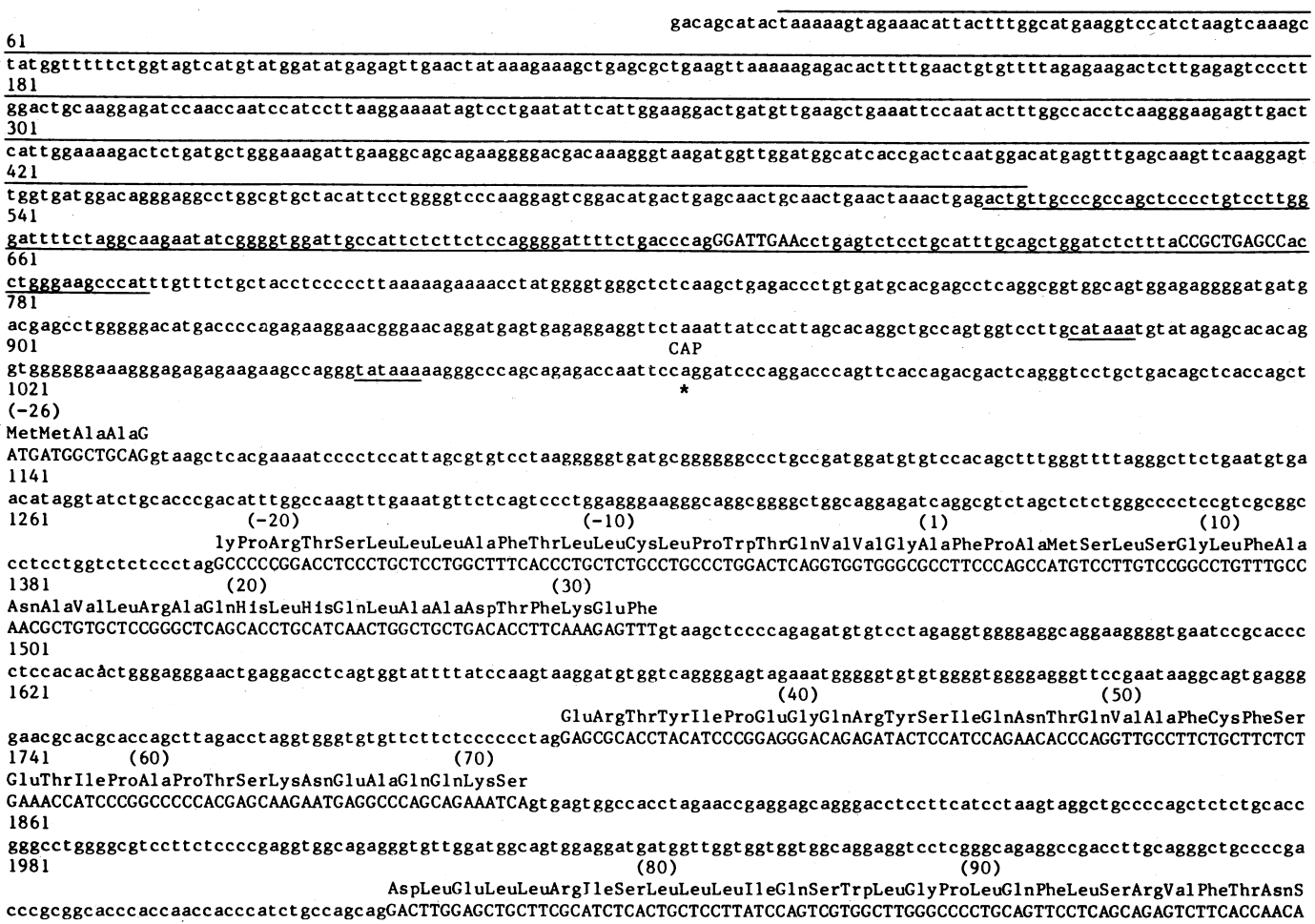


Fig. 1. Restriction enzyme map of genomic clone λOGH2 encoding the ovine growth hormone gene, together with a diagram of the repeated sequence DNA which flanks the gene. Exons are indicated as solid blocks. The nature of the repeated sequence elements is indicated by appropriate letters (see text). A1-A2 indicates the position of a repeat element present in the bovine but not in the ovine gene. Arrows indicate the orientation of the repeated elements.

The structure of the ovine growth hormone gene is similar to that found for other growth hormone genes and, as expected, is very homologous to the bovine gene (Woychik *et al.* 1982; Gordon *et al.* 1983). By means of primer extension studies (Hudson and Davidson 1984), we have shown that the start of transcription is the A residue 58 bases upstream from the start of translation (Fig. 3). Thus, the primary transcript of the gene is 1792 bases in length, consisting of five exons, with intron sizes of 246 bp, 231 bp, 227 bp and 273 bp. At the 5′ end of the gene, a TATAAA sequence is located at position −30, a CATAAAT sequence at position −84 and, starting at position −142, a 41 bp sequence which is highly conserved in bovine, rat and human growth hormone genes. At the 3′ end of the gene, an AATAAA polyadenylation signal is present at position 2734, with the polyadenylation site, determined from the growth hormone cDNA sequence, located at position 2758.

The amino acid sequence of the mature hormone deduced from the nucleotide sequence of the gene differs at only one amino acid compared with the published protein sequence of Li *et al.* (1973). A 26 amino acid leader peptide can also be identified from the nucleotide sequence.

It was known from amino acid sequence data that the mature ovine hormone is very similar to that of the bovine growth hormone (Wallis 1978). We observed only three amino acid differences between the two hormones. In the leader peptide sequence, amino acid 12 is threonine in the ovine sequence and alanine in the bovine sequence. In the mature protein, amino acid 130 is valine in the ovine protein and glycine in the bovine hormone. Amino acid 99, which has been reported to be aspartic acid in the ovine protein (Li *et al.* 1973) was found to be asparagine in the nucleotide-derived sequence. The amino acid in this position in the bovine hormone is also asparagine and, since there is no significant difference in charge between the two hormones when examined by electrophoresis (Wallis 1978), it is probable that the nucleotide-derived sequence is correct.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(60)
(70)
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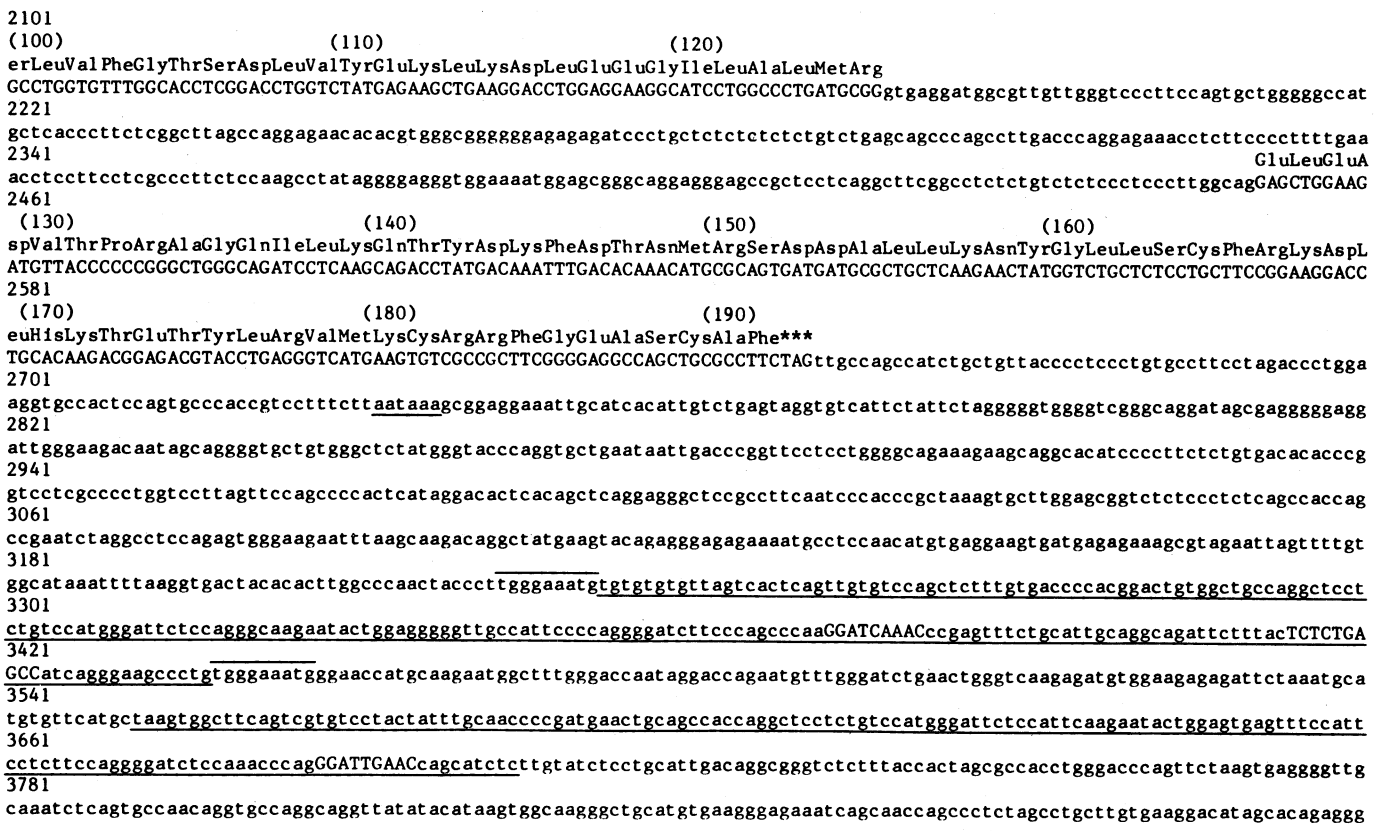

Fig. 2. Nucleotide sequence of the ovine growth hormone gene and flanking repeated sequence DNA. The CAP site is indicated by *, and the TATA box, CAT sequence and polyadenylation signal are underlined. Exons are in upper case and introns in lower case. Within the 5′ and 3′ flanking DNA, repeated sequence elements are indicated by underlining and overlining and correspond with the open boxed regions of Fig. 1. RNA polymerase III promoter elements are in upper case. The 9 bp direct repeats of the C-A3 element in the 3′ flanking DNA are overlined.

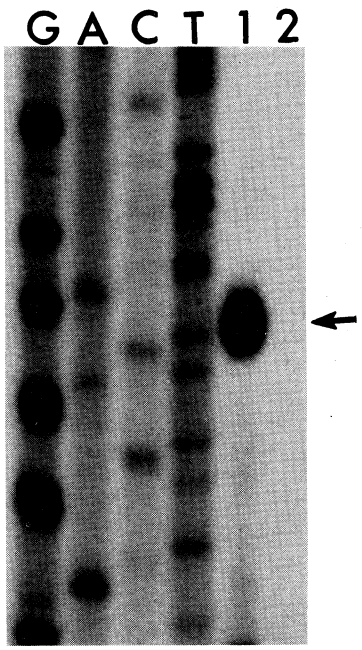

Fig. 3. Determination of the start of transcription of the ovine growth hormone gene by primer extension analysis. A *Pst*I/*Hin*fI primer fragment was prepared from an M13 clone using the prime, cut technique of Hudson and Davidson (1984). Lane 1 shows the primer extension product, against which is shown the relevant DNA sequence of the corresponding M13 clone. Lane 2 is a negative control.

A strong sequence homology was also maintained at the nucleotide level between the ovine and bovine (Miller *et al.* 1980) cDNAs. Only three base changes were observed between the two 5′ untranslated regions, 15 base changes between the two coding portions, and four base changes and a six base bovine sequence deletion in the 3′ untranslated regions.

The ovine growth hormone gene is flanked by artiodactyl-specific middle-repetitive DNA. One such family of sequences was identified by comparison of the growth hormone DNA with the consensus sequence for a family of repetitive elements found in the goat β-globin gene (Spence *et al.* 1985) and the bovine corticotropin–lipotropin precursor gene (Watanabe *et al.* 1982). This family has been named the 'C-A3' family (Rogers 1985). The homology between the sheep sequences and the consensus sequence is high, ranging from 76% to 83%.

At the 3′ end of the gene, the first repeated sequence element is located 476 bp downstream from the polyadenylation site (Fig. 1). It also has the overall structure of a C-A3 repeated element. It is 206 bp in length, bounded by 9 bp direct repeats and contains within the C element an RNA polymerase III split promoter (Fig. 2). Further downstream is a short truncated sequence, consisting of an A3 element and part of a C-element, but no apparent flanking direct repeats. This suggests the occurrence of a post-insertional deletion, removing part of the C element and adjacent DNA.

A third C-A3 type element is found beginning just 290 bp 5′ to the transcription initiation site. This sequence is deleted in the A region. Interestingly, examination of the DNA sequence of the bovine growth hormone gene revealed the presence of this same C-A3 element 5′ to the bovine gene. However, DNA sequences 5′ to the bovine and ovine genes differ, due to the presence of an 'A1-A2' type repetitive element (Rogers 1985) 5′ to the bovine gene. The insertion has occurred on the C border of the common C-A3 element (Fig. 1) and indicates that repetitive DNA sequences begin just 292 bp 5′ to the transcription initiation site of the bovine gene, starting with an intact A1-A2 element, followed by the C-A3 element common to sheep and cattle.

Immediately adjacent to the truncated A3 element in the sheep gene lies a copy of a previously unreported class of artiodactyl middle-repetitive DNA. We have named this an E element to distinguish it from the known artiodactyl sequences. Its relative position is shown in Fig. 1 and its sequence is shown in Fig. 4 (sequence 1). The 493 bp element was identified by comparing

sheep growth hormone DNA with other sheep genomic sequences (B. W. Wilson, K. E. Edwards, C. R. Byrne and K. A. Ward, unpublished) (Fig. 4, sequences 2, 3), and a similarity search on the GenBank nucleic acid sequence data bank (rel. 44.0) revealed the presence of the same element in a pair of related goat β-globin pseudogenes (Cleary *et al.* 1981). By comparing the sequences of five E elements (Fig. 4), we conclude that they have no obvious RNA polymerase III promotor elements, but do appear to be related to the A sequences of the previously characterized C-A3 and A1-A2 elements, since they have 80 bp of sequence in common.

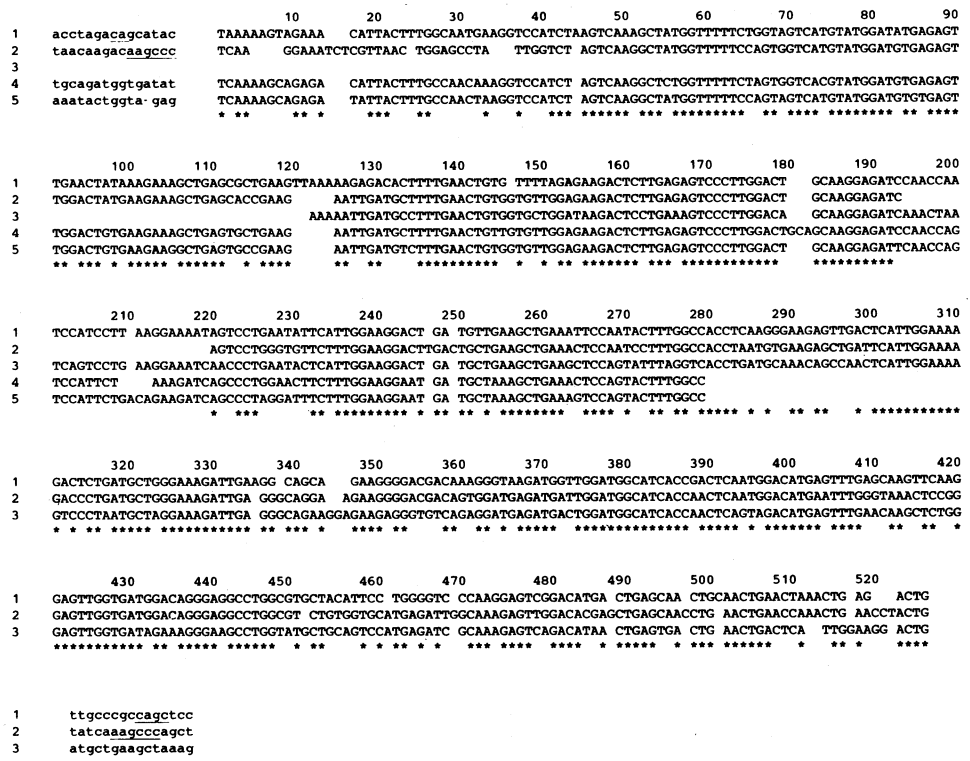

Fig. 4. Comparison of five artiodactyl repeated sequence elements (see text). Sequences 1–3 are obtained from ovine DNA, and sequences 4 and 5 are from goat DNA (Cleary *et al.* 1981). Repeated sequences are in upper case and flanking DNA in lower case. Possible direct repeats in the flanking DNA of sequences 1 and 2 are underlined.

Genomic DNA Hybridization

The sequences in the sheep genome which hybridize with the sheep GH cDNA were examined on Southern blots of *Eco*RI, *Bam*HI and *Hin*dIII restriction digests of sheep DNA. The results obtained varied between animals. In the DNA sample analysed in Fig. 5 (*left*), single bands hybridized to the probe in each digest, their sizes being 7.0 kbp (*Eco*RI), 10.1 kbp (*Hin*dIII) and 8.3 kbp (*Bam*HI). This supports the view that the sheep contains a single growth hormone gene. However, in Fig. 5 (*right*), two different samples of sheep DNA are compared. In each sample, *Bam*HI and *Hin*dIII digests each give rise to two bands of hybridization, while *Eco*RI digests give rise to a single 12.5 kbp band in one sample, and two bands of 12.5 and 7.0 kbp in the other sample. One possible explanation is that there is restriction fragment length polymorphism due to mutations in the DNA flanking the growth hormone gene.

Discussion

The gene sequences for rat, human and bovine growth hormones have been elucidated in the past few years (Barta *et al.* 1981; DeNoto *et al.* 1981; Woychik *et al.* 1982; Gordon *et*

al. 1983). In each, the coding sequence is divided into five exons, and the introns in general are about 200-300 bp in length. The ovine gene fits this general structure. A particularly high homology has been found with the bovine gene. This is not very surprising in view of the short period of evolutionary divergence between the two species (Dayhoff 1972).

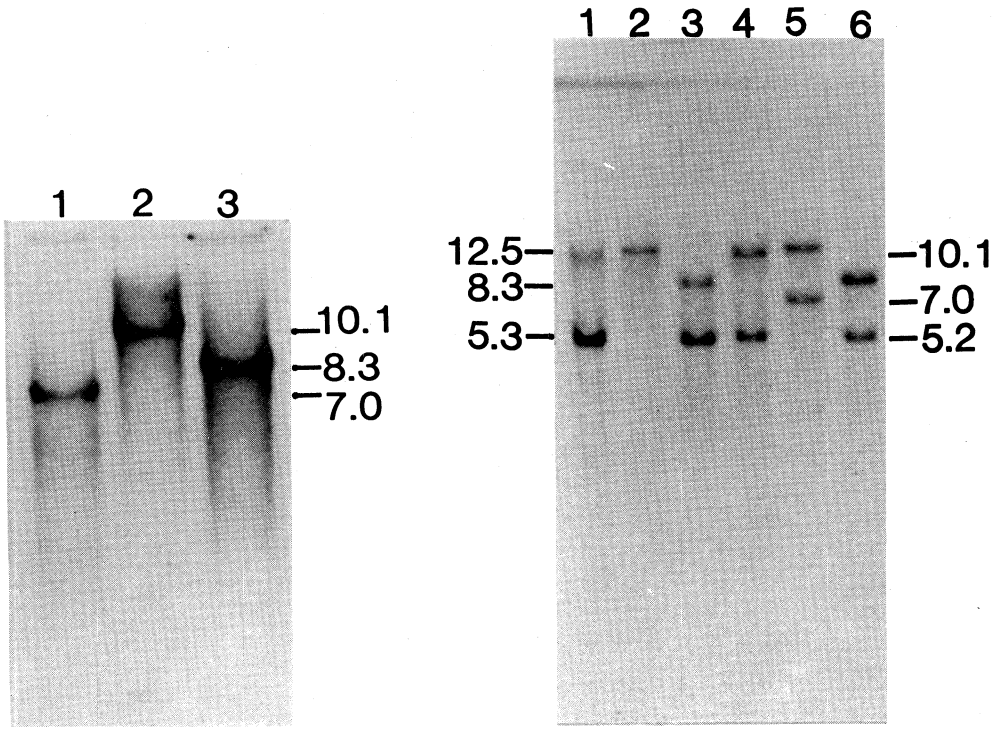

Fig. 5. Southern blots of restriction enzyme digests from two different sheep genomic DNA samples, hybridized with a probe consisting of the insert from clone λOGH1. (*Left*) DNA from sheep A. Sample 1 digested with *Eco*RI, 2 with *Bam*HI and 3 with *Hin*dIII. (*Right*) Digests 1–3 are from sheep B, digests 4–6 are from sheep C. Samples 1 and 4 were digested with *Bam*HI, 2 and 5 with *Eco*RI, 3 and 6 with *Hin*dIII. Sizes of indicated bands are kilobase pairs.

The finding that the ovine gene is bounded by middle-repetitive DNA is consistent with similar findings for the human and rat genes (Miller and Eberhardt 1983). The function, if any, of these sequences remains unknown, but the strong homology of the C-element to eukaryotic tRNAs, the presence of a strong RNA polymerase III promoter sequence, and the formation of terminal repeats during insertion suggest that they are a form of transposable element, or 'retroposon' as defined by Rogers (1985). They might, therefore, provide a mechanism for the movement or duplication of genes within the genome. The newly found E element located upstream from the sheep growth hormone gene has structural similarities to the previously described A elements of cows and goats. The last 80 bp of this element (as shown in Fig. 4) is homologous to the last 80 bp of the A portion of the C-A3 element, suggesting an evolutionary relationship.

If we assume that the repeated sequence elements provide upstream and downstream boundaries for the ovine growth hormone gene, then the gene, and its associated regulatory elements, are included in a region starting 290 bp upstream from the transcription initiation site and ending 476 bp downstream from the polyadenylation site. The regulatory sequences would then be confined to the 5′ flanking 290 bp of sequence, the introns, or the 476 bp 3′ flanking region. The identification of repeated elements 292 bp upstream from the transcription initiation

site of the bovine growth hormone gene suggests that the overall size of the bovine gene is similar to the ovine gene, but insufficient sequence is available in the 3′-flanking region of the bovine gene to be sure of this. Nevertheless, the similar size and the first repeated element (290 bp for the ovine gene and 292 bp for the bovine gene) suggests that this portion of DNA must be maintained intact, and hence contains information essential for the function of both genes.

5′ flanking DNA in the rat growth hormone gene has been the subject of recent work from a number of laboratories. Results gained from transfection studies using a rat growth hormone–neomycin resistance gene construct demonstrated that a region of DNA extending just 235 bp upstream from the cap site of the rat gene is sufficient to direct cell-type specific and thyroid hormone regulated transcription of the rat growth hormone gene (Crew and Spindler 1986), although sequences required for full responsiveness to thyroid hormone are located further 5′. Similarly, Nelson *et al.* (1986) used transfection studies with a growth hormone–chloramphenicol acetyltransferase gene construct to demonstrate the existence of a cell-type specific enhancer located between 47 and 235 base pairs 5′ to the rat growth hormone gene. This agrees with preliminary results of Baxter *et al.* (1986), who located the tissue-specific element of the rat growth hormone gene within a region of DNA 234 base pairs 5′ to the cap site.

Acknowledgments

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